



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/50, A61K 38/31	A1	(11) International Publication Number: WO 96/35950 (43) International Publication Date: 14 November 1996 (14.11.96)
(21) International Application Number: PCT/EP96/02064 (22) International Filing Date: 11 May 1996 (11.05.96) (30) Priority Data: 08/440,061 12 May 1995 (12.05.95) US (71) Applicant: UNIVERSITY OF BUCKINGHAM [GB/GB]; Hunter Street, Buckingham MK18 1AG (GB). (72) Inventors: DUNMORE, Simon, Jon; University of Bucking- ham, Hunter Street, Buckingham MK18 1AG (GB). DAV- ENPORT, Michelle; University of Buckingham, Hunter Street, Buckingham MK18 1AG (GB). CAWTHORNE, Michael, Anthony; University of Buckingham, Hunter Street, Buckingham MK18 1AG (GB). (74) Agents: COCKBAIN, Julian et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: INHIBITION OF AMYLIN RELEASE (57) Abstract A method of determining the ability of a compound to both bind to somatostatin type-5 receptor ("SSTR-5") and inhibit amylin release. The method includes obtaining a preparation, either a cell preparation or a membrane preparation, which contains SSTR-5; incubating the preparation, the compound, and an SSTR-5 ligand, at least one of the ligand and the compound being detectably labeled; determining the ability of the compound to compete against the ligand for binding to SSTR-5; if and only if the compound is determined to be able to bind to SSTR-5, obtaining pancreatic cells; incubating the compound, the pancreatic cells, and an amylin release stimulator under conditions in which the amylin release stimulator would induce release of amylin from the pancreatic cells; and determining the ability of the compound to inhibit amylin release. Also disclosed is a method of treating hyperamylinemia with a ligand selective for SSTR-5.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein*	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

INHIBITION OF AMYLIN RELEASE

5 Background of the Invention

Amylin, a 37-amino acid polypeptide structurally related to calcitonin gene-related protein (see Cooper, Endocrine Review 15:163 (1994)), is primarily
10 synthesized, packaged, and secreted from the β -cells of pancreatic islets. Apart from the islet of Langerhans, amylin-like immunoreactivity activity has also been detected in lung, gastrointestinal tract, and the nervous system. See Miyazato M. et al., Bioch. Bioph. Res. Comm. 181:293 (1991); Chance et al., Brain Res. 539:352 (1991); Mulder et al., Gastroenterology 107:712 (1994).

The presence of an abnormally high concentration of amylin in the blood, i.e. hyperamylinemia, has been
20 found in patients with pancreatic cancer (see Permert et al., N. Engl. J. Med 330:313 (1994)), obese patients (see Huang et al., Hypertension 19 (Supp. I):101 (1992)), and in prediabetic patients (see Erickson J., Diabetologia 35:291 (1992)). The hyperamylinemic state
25 has been associated with both diabetes as well as amyloid formations (see Dekoning et al., Proc. Natl. Acad. Sci. USA 91:8467 (1994)). Amyloid formation causes destruction of β -islet cells and eventually pancreatic dysfunction (see Johnson et al., Lab. Invest. 66:522 (1992) and Lorenzo et al., Nature 368:756 (1994)).

Native somatostatin is comprised of both a 14-amino acid isoform (somatostatin-14) and a 28-amino acid isoform (somatostatin-28) (see Reichlin, New Eng. J. Medicine 309(24):1495 (1983)). Five distinct
35 somatostatin receptors have been identified and characterized (see Hoyer et al., Naunyn-Schmiedeberg's

Arch. Pharmacol. 350:441 (1994)). Somatostatin produces a variety of effects, including modulation of hormone release, e.g. growth hormone, glucagon, insulin, amylin, and neurotransmitter release. Some of these effects have been associated with its binding to a specific somatostatin receptor. For example, the inhibition of growth hormone has been attributed to somatostatin type-2 receptor ("SSTR-2") (see Raynor et al., Molecular Pharmacol. 43:838 (1993) and Lloyd et al., Am. J. Physiol. 268:G102 (1995)). Because of the short half-life of the native somatostatin, various somatostatin analogs have been developed, e.g. for the treatment of acromegaly (see Raynor et al., Molecular Pharmacol. 43:838 (1993)).

Summary of the Invention

The present invention is based on the discovery that ligands selective for somatostatin type-5 receptor ("SSTR-5") are effective in inhibiting release of amylin from pancreas cells.

An aspect of this invention relates to a method of determining the ability of a compound to both bind to somatostatin type-5 receptor and inhibit amylin release from amylin-secreting pancreas cells. The method includes the steps of (i) obtaining a preparation, either a cell preparation or a membrane preparation, which contains SSTR-5; (ii) incubating the preparation, the compound, and a SSTR-5 ligand, at least one of the ligand and the compound being detectably labeled; (iii) determining the ability of the compound to compete against the ligand for binding to SSTR-5; (iv) if and only if the compound is determined to be able to bind to SSTR-5, obtaining amylin-secreting pancreatic cells (e.g. cells in an intact pancreas from a rodent such as a rat or a mouse, pancreatic islet cells such as β cells, or amylinoma cells); (v) incubating the compound,

the pancreatic cells, and an amylin release stimulator (e.g. glucose or D-glyceraldehyde) under conditions in which the amylin release stimulator would induce release of amylin from the pancreatic cells; and (vi)
5 determining the ability of the compound to inhibit amylin release.

By "SSTR-5 ligand" is meant a compound which binds to SSTR-5, e.g. somatostatin-14, somatostatin-28, an analog of somatostatin-14 or somatostatin-28 (such as
10 [Tyr¹¹]-somatostatin-14), or an antibody raised against SSTR-5. Either the ligand or the test compound can be labeled with a radioactive isotope or a nonradioactive (e.g. fluorescent, chemiluminescent, or biotinylated) molecule. Preferably, [¹²⁵I-Tyr¹¹]-somatostatin-14 is
15 used as a labeled SSTR-5 ligand to practice the above-described method. Other examples of labeled ligands include ¹²⁵I-LTT-somatostatin-28 (see Patel *et al.*, Endocrinol. 135(6): 2814 (1994)) and ¹²⁵I-CGP 23996 (see Raynor *et al.*, Mol. Pharm. 44:385-392 (1993)).

20 Examples of the pancreatic cells which are used to practice the above method include both rodent and human islet cells (e.g. β and δ cells) and pancreatic tumor cells (e.g. amylinoma cells). The pancreatic cells can be incubated either in vitro or in vivo. Examples of an
25 in vitro system include an isolated rat pancreas, the rat β -cell line RINm5f, and the hamster β -cell line HIT-T15. As an example of an in vivo system, Sprague-Dawley or Zucker fatty rats can be used as animal models to test the amylin inhibition activity of test somatostatin
30 analogs. An amylin release stimulator, e.g. 16.7 mM glucose, can be injected into the animal. The test somatostatin analog is then injected into the animals at various concentrations. Blood samples can be taken from the animal, and the amount of amylin present before and
35 after the injection of the test somatostatin analog can be determined by radioimmunoassay.

By "amylin release stimulator", is meant a compound

which stimulates the release of amylin stored in pancreatic cells. Examples of amylin release stimulators include glucose, D-glyceraldehyde, or L-arginine.

5 The method described above can be used to screen for new compounds capable of inhibiting amylin release from the pancreas in a patient. To promote efficiency, when the method is used in a screening project, two or more test compounds can be linked together as a single
10 sample, and, if necessary, subsequently divided and retested.

 Another aspect of this invention relates to a method of treating (i.e. either preventing or ameliorating) hyperamylinemia by inhibiting release of
15 amylin from the pancreas in a subject (e.g. a warm blooded animal such as a mammal, e.g. a human being) who suffers from, or is predisposed to suffer from, hyperamylinemia. The method includes administering
(e.g. parenterally, intravenously, subcutaneously,
20 transdermally, intramucously, or via implantation of a sustained release formulation), to the subject an amount of a SSTR-5 agonist, the amount being effective in treating hyperamylinemia, i.e. in lowering amylin levels in the bloodstream by inhibiting the release of amylin
25 from pancreas cells.

 What is meant by "SSTR-5 agonist" or, in the claims, "a somatostatin type-5 receptor agonist" is a compound which (i) is more selective for SSTR-5 than for SSTR-2, i.e. its K_i for SSTR-5 is lower than that for
30 SSTR-2 (as determined by one of the receptor binding assays described in the working examples below); and (ii) inhibits the release of amylin from pancreas cells induced by an amylin release stimulator (as determined by one of the functional assays described in the working
35 examples below). Both examples of SSTR-5 agonists and the procedures of selecting them appear in the "Description of the Invention" section below.

The above-described therapeutic method can be used to treat a subject suffering from pancreatic cancer, prediabetic symptoms, or non-insulin dependent diabetes.

An effective amount depends upon the conditions
5 being treated, the route of administration chosen, and the specific activity of the compound used, and ultimately will be decided by the attending physician or veterinarian. While it is possible for a SSTR-5 agonist to be administered as the pure or substantially pure
10 compound, it is preferable to present it as a pharmaceutical formulation or preparation. The formulations to be used in the present invention, for both humans and animals, comprise any of the SSTR-5 agonists to be described below, together with one or
15 more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The carrier must be "acceptable" in the sense of being compatible with the active ingredient(s) of the formulation (and preferably, capable of stabilizing peptides) and not deleterious to
20 the subject to be treated.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods
25 include the step of bringing the active ingredient(s) into association with the carrier which constitutes one or more accessory ingredients.

In general, the formulations for tablets or powders are prepared by uniformly and intimately blending the active ingredient with finely divided solid carriers,
30 and then, if necessary as in the case of tablets, forming the product into the desired shape and size.

Formulations suitable for intravenous or subcutaneous administration, on the other hand, conveniently comprise sterile aqueous solutions of the
35 active ingredient(s). Preferably, the solutions are isotonic with the blood of the subject to be treated. Such formulations may be conveniently prepared by

dissolving solid active ingredient(s) in water to produce an aqueous solution, and rendering the solution sterile. The formulation may be presented in unit or multi-dose containers, for example sealed ampoules or vials. Delivery of drug by parenteral implantation of a sustained release formulation is well-known in the art. See for example U.S. Patent No. 3773919, U.S. Patent No. 4767628; and PCT Application No. WO 94/00148.

Also within the scope of this invention are a SSTR-5 agonist for use in therapy or prophylaxis, e.g. in treating a disease or a disorder relating to hyperamylinemia, and the use of a SSTR-5 agonist for the manufacture of a medicament for the treatment of such disease or disorder. The invention further relates to novel SSTR-5 agonists identified by the method of the invention and to pharmaceutical compositions containing SSTR-5 agonists.

Use of a ligand more selective for SSTR-5 than for SSTR-2 to treat hyperamylinemia minimizes undesirable side effects.

Other features and advantages of the invention will be apparent from the following drawings and detailed description of several embodiments and from the appended claims.

Brief Description of the Drawings

The drawings are first briefly described:

Fig. 1 is a graph showing the effect of somatostatin-14 on glucose-induced amylin secretion.

Fig. 2 is a graph showing the effect of a somatostatin analog on glucose-induced amylin secretion.

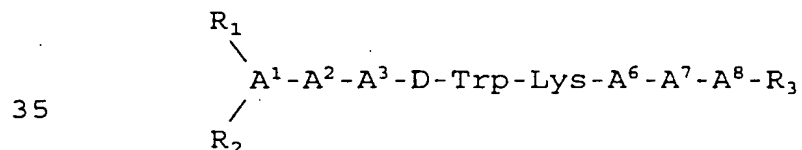
Fig. 3 is a graph showing the effect of another somatostatin analog on glucose-induced amylin secretion.

Description of the Invention

A SSTR-5 agonist which can be used to practice the therapeutic method of the present invention is a

somatostatin receptor ligand which is more selective for SSTR-5 than for SSTR-2. Selectivity of a SSTR-5 agonist for a particular receptor is determined by receptor binding assays in which its respective binding inhibition constants (K_i 's) for SSTR-5 and SSTR-2 are determined. The SSTR-5 agonists which are capable of inhibiting amylin secretion are those compounds having either (i) a K_i for the human SSTR-5, as determined by utilizing CHO-K1 cells transfected with the human SSTR-5, which is lower than that for the human SSTR-2, as determined by utilizing CHO-K1 cells transfected with the human SSTR-2, or (ii) a K_i for the rat SSTR-5, as determined by utilizing rat olfactory bulb cells, which is lower than that for the rat SSTR-2, as determined by utilizing AR42J rat pancreatic acinar tumor cells. The just-mentioned four binding assays are described in detail in the working examples below. A preferred SSTR-5 agonist is at least 3 times as selective for SSTR-5 as for SSTR-2; in other words, its ratio of K_i for SSTR-2 to K_i for SSTR-5 is 3 or higher (e.g. 3 to 10000, for example 3 to 1000, particularly 3 to 100, more particularly 3 to 60) in either the human system or the rat system. An even more preferred SSTR-5 agonist has a selectivity for SSTR-5 which is 10 times or higher than that for SSTR-2.

Preferably, the above-mentioned SSTR-5 agonists are linear peptides, for example oligopeptides such as octapeptides. Examples of such linear peptides include, but are not limited to, those covered by the following generic formula:



wherein

A^1 is a D- or L- isomer of Ala, Leu, Ile, Val, Nle,

Thr, Ser, β -Nal, β -Pal, Trp, Phe, 2,4-dichloro-Phe, pentafluoro-Phe, p-X-Phe, or o-X-Phe, wherein X is CH₃, Cl, Br, F, OH, OCH₃, or NO₂;

5 A² is Ala, Leu, Ile, Val, Nle, Phe, β -Nal, pyridyl-Ala, Trp, 2,4-dichloro-Phe, pentafluoro-Phe, o-X-Phe, or p-X-Phe, wherein X is CH₃, Cl, Br, F, OH, OCH₃, or NO₂;

 A³ is pyridyl-Ala, Trp, Phe, β -Nal, 2,4-dichloro-Phe, pentafluoro-Phe, o-X-Phe, or p-X-Phe, wherein X is CH₃, Cl, Br, F, OH, OCH₃, or NO₂;

10 A⁶ is Val, Ala, Leu, Ile, Nle, Thr, Abu, or Ser;

 A⁷ is Ala, Leu, Ile, Val, Nle, Phe, β -Nal, pyridyl-Ala, Trp, 2,4-dichloro-Phe, pentafluoro-Phe, o-X-Phe, or p-X-Phe, wherein X is CH₃, Cl, Br, F, OH, OCH₃, or NO₂;

15 A⁸ is a D- or L- isomer or Ala, Leu, Ile, Val, Nle, Thr, Ser, Phe, β -Nal, pyridyl-Ala, Trp, 2,4-dichloro-Phe, pentafluoro-Phe, p-X-Phe, or o-X-Phe, wherein X is CH₃, Cl, Br, F, OH, OCH₃, or NO₂;

20 each R₁ and R₂, independently, is H, lower acyl (e.g. up to C₇ acyl) or lower alkyl (e.g. C₁ to C₆ alkyl); and R₃ is OH or NH₂; provided that at least one of A¹ and A⁸ and one of A² and A⁷ must be an aromatic amino acid; and further provided that A¹, A², A⁷, and A⁸ cannot all be aromatic amino acids.

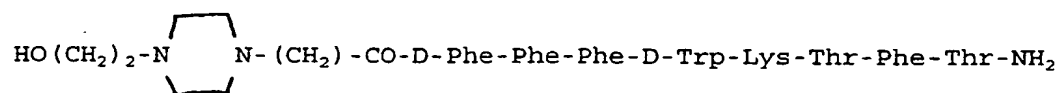
25 Examples of linear SSTR-5 agonists to be used in the therapeutic method of this invention include:

 H-D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH₂ (BIM-23052);
 H-D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-OH (Analog I);
 H-D-Phe-p-chloro-Phe-Tyr-D-Trp-Lys-Thr-Phe-Thr-NH₂;
 H-D-Phe-p-NO₂-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH₂;
30 H-D-Nal-p-chloro-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH₂;
 H-D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH₂;
 H-D-Phe-p-chloro-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH₂;
 H-D-Phe-Ala-Tyr-D-Trp-Lys-Val-Ala-D- β -Nal-NH₂; and
 H-D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Phe-NH₂.

35 If desired, one or more chemical moieties, e.g. a sugar derivative, mono or poly-hydroxy C₂₋₁₂ alkyl, mono or poly-hydroxy C₂₋₁₂ acyl groups, or a piperazine

- 9 -

derivative, can be attached to a SSTR-5 agonist, e.g. to the N-terminus amino acid (see PCT Application WO 88/02756, European Patent Application 0 329 295, and PCT Application WO 94/08875). An example of a SSTR-5
5 agonist which contains an N-terminal chemical substitution is:



10

(Analog II)

Note that, unless indicated otherwise, for all amino acid sequence formulas described herein, each amino acid residue, e.g. A¹ or Lys, represents the structure of NH-C(R)H-CO-, in which R is the side chain. Lines between amino acid residues represent peptide bonds which join two amino acids. Also, where the amino acid residue is optically active, it is the L-form configuration that is intended unless D-form is expressly designated. The uncommon abbreviations β -Nal, β -Pal, Nle, and Abu stand for, respectively, 3-(β -naphthyl)alanine, 3-(β -pyridyl)alanine, norleucine, and α -aminobutyric acid.

Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. Indeed, unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Synthesis of somatostatin receptor ligands

Synthesis of short amino acid sequences is well established in the peptide art. For example, synthesis of BIM-23052, the structure of which is described above, can be achieved by following the protocol set forth in Example I of European Patent Application 0 395 417 A1. The synthesis of somatostatin analogs with a substituted N-terminus can be achieved, for example, by following the protocol set forth in WO 88/02756, European Patent Application No. 0 329 295, and PCT Application No. WO

94/08875.

SSTR-5 agonists such as Analogs I and II can be synthesized in an similar manner.

5 Synthesis of radiolabeled somatostatin receptor ligands

 The synthesis of radiolabeled somatostatin receptor ligands is well documented and is within the ability of a person of ordinary skill in the art (see for example Czernick et al., J. Biol. Chem. 258:5525 (1983)). For
10 example, the SSTR-2 radioligand, cyclo (N-Me-Ala-Tyr[¹²⁵I]-D-Trp-Lys-Val-Phe) or [¹²⁵I-Tyr]MK-678, was synthesized in the manner set forth below: The
uniodinated cyclic hexapeptide was first synthesized according to the procedure set forth in U.S. Patent No.
15 4310518. The hexapeptide was then iodinated utilizing the chloramine-T method. More specifically, 50 nM of sodium phosphate buffer (pH 7.4, 50 μ l), a solution of MK-678 (1 mM in phosphate buffer, 10 μ l), and Na¹²⁵I (1 mCi in approximately 10 μ l of H₂O) were added to a
20 polypropylene tube. The reaction was started by addition of a freshly prepared chloramine-T solution (1 mg/ml in H₂O; 10 μ l). The mixture was agitated for 30 sec and then a solution of cysteine in phosphate buffer (2 mg/ml; 100 μ l) was added to stop the reaction. The
25 material was purified by HPLC. Fractions containing the desired product were identified by UV detection at 214 nM on a 20-50% gradient for 30 min, pooled, diluted with 10% ethanol, and stored at -20°C.

 Another radiolabeled somatostatin receptor ligand
30 [¹²⁵I-Tyr¹¹]somatostatin-14 can be prepared following a similar procedure. [¹²⁵I-Tyr¹¹]somatostatin-14 is also commercially available.

Somatostatin receptor binding assays

35 (1) Rat SSTR-2 Binding Assay

 Crude membranes were prepared by homogenizing AR42J cells (ATCC, Rockville, Maryland, USA; ATCC No. CRL1992)

- 12 -

in 20 ml of ice-cold 50 mM Tris-HCl (Buffer A) with a POLYTRON homogenizer (Brinkmann Instruments, Westbury, New York, USA) at setting 6, for 15 sec. Additional Buffer A was added to obtain a final volume of 40 ml, and the homogenate was centrifuged in a Sorval SS-34 rotor (DuPont, Newtown, Connecticut, USA) at 39,000 g for 10 min at 0-4°C. The resulting supernatant was decanted and discarded. The pellet was rehomogenized in ice-cold Buffer A, diluted, and centrifuged as before. The final pellet was resuspended in the 10 mM Tris HCl and held on ice for the receptor binding assay.

Aliquots of the membrane preparation were incubated for 90 min at 25°C with 0.05 nM [¹²⁵I-Tyr]MK-678 (2000 Ci/mmol) in 50 mM HEPES (pH 7.4) containing a test peptide of various concentrations (e.g. 10⁻¹¹ to 10⁻⁶ M), 10 mg/ml bovine serum albumin (fraction V) (Sigma Chemical Co., St. Louis, Missouri, USA), MgCl₂ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 mg/ml), and phenylmethylsulphonyl fluoride (0.02 mg/ml). The final assay volume was 0.3 ml. The incubations were terminated by rapid filtration through GF/C filters (pre-soaked in 0.3% polyethylenimine for 30 min) using a filtration manifold (Brandel, Gaithersburg, Maryland, USA). Each tube and filter was then washed three times with 5 ml aliquots of ice-cold Buffer A. Specific binding was defined as the total [¹²⁵I-Tyr]MK-678 bound somatostatin-14 bound minus that bound in the presence of 200 nM somatostatin-14.

The following somatostatin analogs were assayed: somatostatin-14, Analog I (structure shown above), Analog II (structure shown above), BIM-23052 (structure shown above), BIM-23014 (H-D-β-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Ser-NH₂; LANREOTIDE or SOMATULINE), and SMS 201-995 (H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Threoninol; SANDOSTATIN or OCTREOTIDE). The K_i values for these test somatostatin analogs were calculated by using the following formula: $K_i = IC_{50} / [1 + (LC/LEC)]$ where IC₅₀ is

- 13 -

the concentration of test compound required to inhibit 50 percent of the specific binding of the radioligand [I^{125} -Tyr]MK-678, LC is the concentration of the radioligand (0.05 nM), and LEC is the equilibrium dissociation constant of the radioligand (0.155 nM). The K_i values calculated for the tested peptides are shown in the column under the heading "SSTR-2" in Table I.

(2) Rat SSTR-5 Binding Assay

Crude membranes were prepared by homogenization of rat olfactory bulb cells (Zivic-Miller Laboratory, Inc., Zellenople, Pennsylvania, USA) in 20 ml of ice-cold 50 mM Tris-HCl with a POLYTRON homogenizer (setting 6, 15 sec). Buffer was added to obtain a final volume of 40 ml, and the homogenate was centrifuged in a Sorval SS-34 rotor at 39,000 g for 10 min at 0-4°C. The resulting supernatant was decanted and discarded. The pellet was rehomogenized in ice-cold buffer, diluted, and centrifuged as before. The final pellet was resuspended in the 10 mM Tris HCl and held on ice for the receptor binding assay.

Aliquots of the membrane preparation were incubated for 30 min at 30°C with 0.05 nM [I^{125} -Tyr¹¹]somatostatin-14 (2000 Ci/mmol; Amersham Corp., Arlington Heights, Illinois, USA) in 50 mM HEPES (pH 7.4) containing a test peptide of various concentrations (e.g. 10^{-11} to 10^{-6} M), 10 mg/ml bovine serum albumin (fraction V) (Sigma Chemical Co., St. Louis, Missouri, USA), $MgCl_2$ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 mg/ml), and phenylmethanesulphonyl fluoride (0.02 mg/ml). The final assay volume was 0.3 ml. The incubations were terminated by rapid filtration through GF/C filters (pre-soaked in 0.3% polyethylenimine for 30 min) using a Brandel filtration manifold. Each tube and filter were then washed three times with 5 ml aliquots of ice-cold buffer. Specific binding was defined as the total [I^{125} -Tyr¹¹]somatostatin-14 bound minus that bound in the

presence of 1000 nM BIM-23052. The K_i values for the tested somatostatin-14 and its analogs were calculated by using the following formula: $K_i = IC_{50}/[1+(LC/LEC)]$ where IC_{50} is the concentration of test compound required to inhibit 50 percent of the specific binding of the radioligand [^{125}I -Tyr 11] somatostatin-14, LC is the concentration of the radioligand (0.05 nM) - and LEC is the equilibrium dissociation constant of the radioligand (0.16 nM). The K_i values (in nM) for the tested somatostatin analogs are shown in the column under the heading "SSTR5" in Table I.

Table I also shows the respective ratios of the K_i 's for the rat SSTR-2 and the K_i 's for the rat SSTR-5. Thus, those somatostatin analogs with such ratios greater than one (e.g. BIM-23052, Analog I, and Analog II) are more selective for the rat SSTR-5 than for SSTR-2.

TABLE I

COMPOUND	SSTR-2	SSTR-5	SSTR-2/SSTR-5
BIM-23052	8.04	3.16	2.54
Analog I	37.2	3.57	10.4
Analog II	305	5.38	56.7
Somatostatin-14	0.17	0.47	0.34
SMS 201-995	0.38	9.14	0.04
BIM-23014	0.34	64.8	0.005

(3) Human SSTR-2 Binding Assay

The human SSTR-2 cDNA clone has been described (see Yamada *et al.*, Proc. Natl. Acad. Sci. USA. 89:251-255 (1992)) and is available from ATCC (ATCC No. 79046). A 1.7 kilobase BamHI-HindIII fragment containing the entire coding region of the human SSTR-2 receptor has been isolated by restriction endonuclease digestion and is available from New England Biolabs (Beverly,

Massachusetts, USA). This cDNA fragment was inserted into the mammalian expression vector, pCMV (see Russell et al., J. Biol. Chem. 264:8222- 8229 (1989)) using standard molecular biology techniques to produce the expression plasmid, pCMV-human SSTR-2. Other mammalian expression vectors include pcDNA1/Amp (Invitrogen, Sandlesy, California, USA). See for example Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982. The expression plasmid was introduced into the suitable bacterial host, E. Coli HB101 (Stratagene, La Jolla, California, USA) and plasmid DNA, for transfection, was prepared on a Cesium Chloride gradient.

CHO-K1 (ovary, Chinese hamster) cells were obtained from the American Type Culture Collection, Rockville, Maryland, USA (ATCC No. CCL 61). The cells were grown and maintained in Ham's F12 media (Gibco BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum under standard tissue culture conditions.

For transfection, the cells were seeded at a density $1 \times 10^6/60 \text{ cm}^2$ plate (Baxter Scientific Products, McGaw Park, Illinois, USA). DNA mediated transfection was carried out using the calcium phosphate co-precipitation method (see Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1987). The plasmid pRSV-neo (ATCC; ATCC No. 37198) was included as a selectable marker at 1/10 the concentration of the expression plasmid. CHO-K1 clonal cell lines that have stably inherited the transfected DNA were selected for growth in Ham's F12 media containing 10% fetal bovine serum and 0.5 mg/ml of G418 (Sigma Chemical Co., St. Louis, Missouri, USA). The cells were ring-cloned and expanded in the same media for analysis.

Expression of the human SSTR-2 receptor was detected by Northern blot analysis of total RNA prepared from the cells (see Sambrook et al., Molecular Cloning -

A Laboratory Manual, Ed. 2., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989) and by receptor binding using [125 I-Tyr 11]somatostatin-14 as a ligand. Transfected cell lines expressing the human SSTR-2 receptor were clonally expanded in culture and used in the rat SSTR-2 binding protocol described above. The K_i for the test somatostatin analogs are listed in Table II (where LC is 0.05 nM and LEC is 0.15 nM).

10 (4) Human SSTR-5 Binding Assay

The human SSTR-5 cDNA clone has been described in the literature (see Panetta et al., Mol Pharmacol. 45:417-427 (1994); O'Carroll et al., Mol Pharmacol. 46:291-298 (1994); and Yamada et al., Biochem. Biophys. Res. Commun. 195:844 (1993) where Fig. 1 depicts the DNA sequence of the human SSTR-5). Using a sense 5'-oligonucleotide primer, immediately preceeding the start codon (residue 30-50), and an antisense 3'-oligonucleotide primer, immediately preceeding the stop codon (residues 1180-1200), a 1170 base pair fragment comprising the full-length coding sequence of the receptor was obtained by standard Reverse-transcription PCR (see Yamada et al., Biochem. Biophys. Res. Commun. 195:844 (1993) where Fig. 1 depicts the DNA sequence of the human SSTR-5; and Innis et al., PCR Protocols, A Guide to Methods and Applications, Academic Press, 1990). The identity of the cDNA fragment was verified by DNA sequencing using the dideoxy-chain termination method (see Sanger et al., Proc Natl Acad Sci USA. 74:5463 (1977)) with the Sequenase kit (United States Biochemicals, Arlington Heights, Illinois, USA).

The human SSTR-5 cDNA fragment was inserted into a mammalian expression vector, pCMV to generate the expression plasmid pCMV-human SSTR-5 by blunt-end ligation (see for example Maniatis et al., Molecular Cloning- A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982). The

expression plasmid was transfected into CHO-K1 cells as described in Example 3 and clonal cell lines were selected and characterized for the expression of the human SSTR-5 receptor by Northern blot analysis and ligand-binding using [¹²⁵I- Tyr¹¹]somatostatin-14. A cell line, CHO-K1M, was shown to express the human SSTR-5. Membranes from the stably transfected cell line were used in the rat SSTR-5 binding protocol described above. The K_i values (in nM) for the test somatostatin analogs are listed in Table II (where LC is 0.05 nM and LEC is 0.18 nM).

TABLE II

COMPOUND	SSTR-2	SSTR-5	SSTR-2/SSTR-5
BIM-23052	14.1	1.22	11.5
Analog I	14.2	12.4	1.14
Analog II	4.15	1.29	3.22
Somatostatin-14	0.18	0.88	0.20
BIM-23014	1.09	5.2	0.21
SMS 201-995	0.5	7.0	0.07

Amylin release inhibition assay using rat pancreas cells

Sprague-Dawley rats (male, 200-300 g) (Harlan-Olac, Bicester, Oxon, UK) were anaesthetized with sodium pentobarbitone (60 mg/kg). The pancreases of the rats were isolated as previously described in Dunmore *et al.*, J. Endocrinol. 92:15-20 (1982). The pancreases were perfused, as described in Dunmore *et al.*, J. Endocrinol. 137: 375-381 (1993), with modified Krebs-Ringer bicarbonate buffer containing 3% dextran T40 (Pharmacia, Milton Heynes, Bucks, UK) and 1% high purity bovine serum albumin (Sigma, Poole, Dorset, UK). After an initial 15-min period to allow for stabilization, the isolated pancreases were perfused at sequential 10 min. intervals, at a rate of 4.5-5.0 ml/min, with the

following buffer compositions: 1) Buffer 1: Krebs-Ringer bicarbonate buffer containing 5.6 mM glucose (to examine baseline amylin secretion); 2) Buffer 2: Krebs-Ringer bicarbonate buffer containing 16.7 mM glucose (to
5 examine high glucose stimulated secretion); 3) Buffer 3: Krebs-Ringer bicarbonate buffer containing 16.7 mM glucose in the presence of 10 nM of test compound (to examine the activity of test compounds on high glucose stimulated amylin secretion); and 4) Buffer 2: Krebs-
10 Ringer bicarbonate buffer containing 16.7 mM glucose (to re-examine high glucose stimulated amylin secretion in order to assess the reversibility of the activity of the test compounds).

Fractions of the perfusate were collected on ice
15 every minute in the presence of 400 KIU/ml aprotinin (Bayer, Hayward's Heath, W. Sussex, UK). Fractions were stored at -20°C until assay. Perfusates collected were divided into 3 x 1.5 ml aliquots and assayed for amylin by radioimmunoassay as described in Dunmore *et al.*, J.
20 Endocrinol. 137:375 (1993). Amylin was assayed using reagents supplied by Peninsula Laboratories Ltd. (St. Helens, Merseyside, UK). These reagents include ¹²⁵I-labelled amylin, rabbit anti-rat amylin antibody, and rat amylin. Bound amylin was precipitated using goat
25 anti-rabbit second antibody - coupled cellulose (Sac-Cal, IDS, Boldon, Tyne and Wear, UK).

Somatostatin-14, BIM-23014, and BIM-23052 were used as test compounds in the above assay. The results of the assay are presented in Figs. 1-3. In all cases,
30 perfusion of the isolated rat pancreas with 16.7 mM glucose produced a significant increase in amylin secretion. Somatostatin-14, which possesses a high affinity for SSTR-5, inhibited glucose-induced amylin secretion at 10 nM (Fig. 1). Somatostatin analog BIM-
35 23014, which possesses a low affinity for SSTR-5, had no effect at the same concentration (Fig. 2). In sharp contrast, BIM-23052, which possesses a high affinity for

SSTR-5 and a low affinity for SSTR-2, produced a 78% decrease in glucose stimulated amylin secretion at the same concentration (Fig. 3). The above assay can also be performed using rat pancreases isolated from female Zucker fatty rats (Harlan-Olac, Bicester, Oxon, UK).

Amylin release inhibition assays using RINm5f cells

The establishment and culture of the rat β -cell line RINm5f cells has been previously described (see Gazdar et al., Proc. Natl. Acad. Sci. USA. 77:3519 (1980); and Praz et al., Biochem. J. 210:345 (1983)). For an in vitro amylin release inhibition assay, RINm5f cells, passage 19-21 are used. The cells (available from the National Institute of Health, Bethesda, Maryland, USA), are maintained under standard tissue culture conditions in RPMI-1640 medium supplemented with 2% fetal bovine serum and 2 mM of glutamine.

For the assessment of amylin release, cells are seeded at 3×10^5 cells/well in 24-well plates (Baxter Scientific Products, McGaw Park, Illinois, USA). After 36-48 hours in culture at 37°C, the media is removed and replaced with 500 μ l of Krebs-Ringer buffer containing 2 mM glucose, 1 mg/ml BSA (Sigma A-4378, Sigma Chemical Co., St. Louis, Missouri, USA) at a pH of 7.4 for 30 min (see Moore et al., Biochem. Biophys. Res. Commun. 179:1 (1993)). At the end of that period, the buffer is replaced with 1 ml of fresh buffer containing either 15 mM D-glyceraldehyde or 50 mM DL-glyceraldehyde (Sigma Chemical Co., St. Louis, Missouri, USA) in the presence or absence of test compounds. At the end of a two-hour incubation at 37°C, the buffer is recovered, centrifuged at 700 rpm for 5 min, and assayed for amylin content as previously described (see Dunmore et al., J Endocrinol. 137:375 (1993)).

Other Embodiments

The foregoing description has been limited to

specific embodiments of this invention. It will be apparent, however, that variations and modifications may be made to the invention, with the attainment of some or all of the advantages of the invention. Such embodiments
5 are also within the scope of the following claims.

CLAIMS:

1. A method of determining the ability of a compound to both bind to somatostatin type-5 receptor and inhibit amylin release from amylin-secreting pancreas cells,
5 said method comprising:
obtaining a preparation which contains somatostatin type-5 receptor;
incubating said preparation, said compound, and a
10 somatostatin type-5 receptor ligand, at least one of said ligand and said compound being detectably labeled;
determining the ability of said compound to compete against said ligand for binding to somatostatin type-5 receptor;
15 if and only if said compound to be determined is able to bind to somatostatin type-5 receptor, obtaining amylin-secreting pancreatic cells;
incubating said compound, said pancreatic cells, and an amylin release stimulator under conditions in
20 which said amylin release stimulator would induce release of amylin from said pancreatic cells; and
determining the ability of said compound to inhibit amylin release.
- 25 2. A method as claimed in claim 1 wherein said preparation is a cell preparation.
3. A method as claimed in claim 1 wherein said preparation is a membrane preparation.
- 30 4. A method as claimed in any one of claims 1 to 3 wherein said preparation is derived from a rodent olfactory bulb.
- 35 5. A method as claimed in any one of claims 1 to 3 wherein said preparation is derived from CHO-K1 cells transfected with a human somatostatin type-5 receptor.

6. A method as claimed in any one of claims 1 to 5 wherein said pancreatic cells are pancreatic islet cells or amylinoma cells or RINm5f cells.
- 5 7. A method as claimed in claim 6 wherein said pancreatic islet cells are β -cells.
8. A method as claimed in any one of claims 1 to 7 wherein said pancreatic cells are cells in an isolated
10 rodent pancreas.
9. A method as claimed in any one of claims 1 to 8 wherein said ligand is detectably labeled.
- 15 10. Use of a somatostatin type-5 receptor agonist for the manufacture of a medicament for combatting hyperamylinemia.
- 20 11. Use as claimed in claim 10 of a somatostatin type-5 agonist which is at least 3 times as selective for somatostatin type-5 receptor as for somatostatin type-2 receptor.
- 25 12. Use as claimed in claim 10 of a somatostatin type-5 agonist which is at least 10 times as selective for somatostatin type-5 receptor as for somatostatin type-2 receptor.
- 30 13. A method of treating hyperamylinemia in a subject, said method comprising administering to said subject an effective amount of a somatostatin type-5 receptor agonist.
- 35 14. A method as claimed in claim 13 wherein as said somatostatin type-5 agonist is used an agonist which is at least 3 times as selective for somatostatin type-5 receptor as for somatostatin type-2 receptor.

15. A method as claimed in claim 13 wherein as said somatostatin type-5 agonist is used an agonist which is at least 10 times as selective for somatostatin type-5 receptor as for somatostatin type-2 receptor.

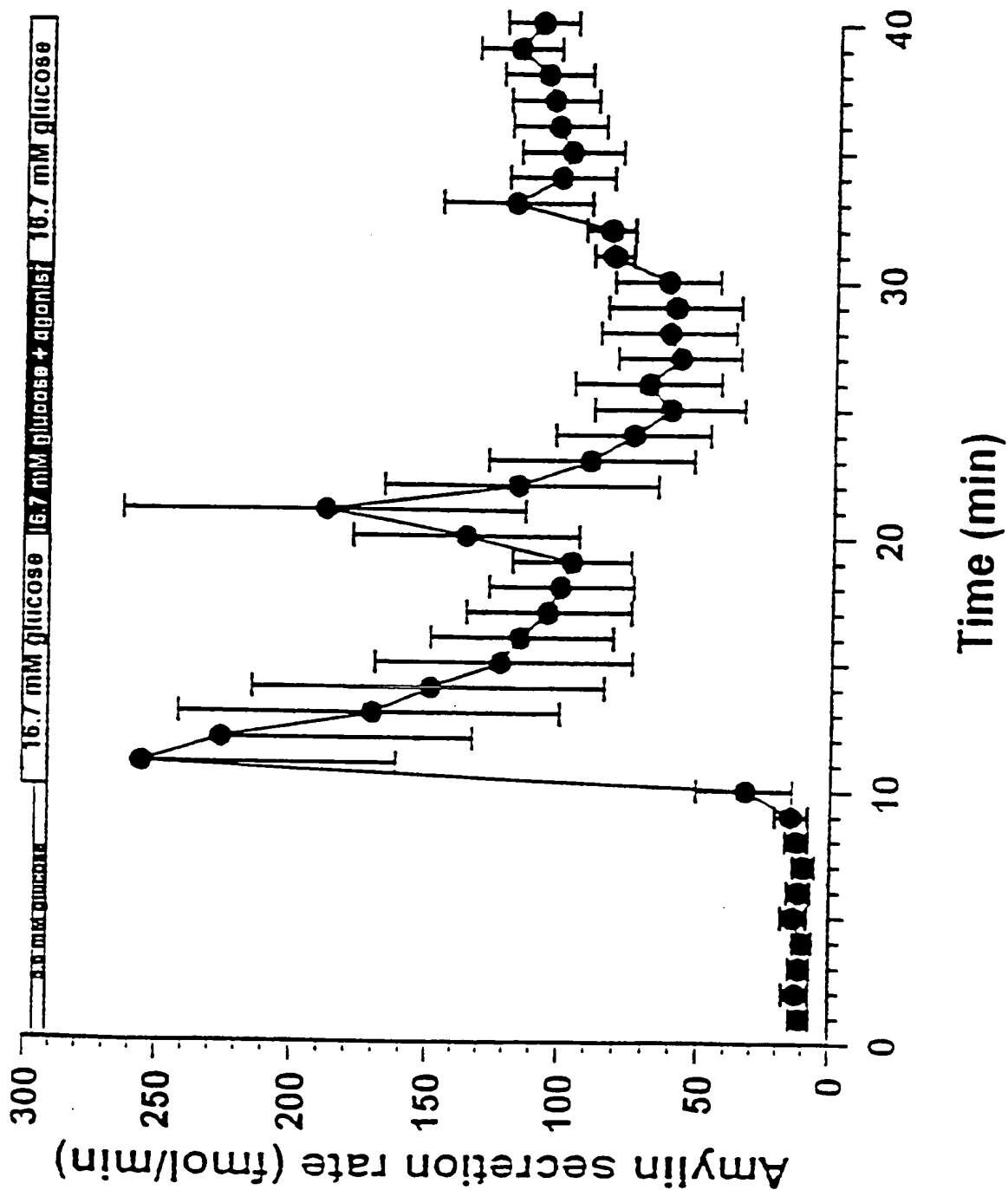


Fig. 1

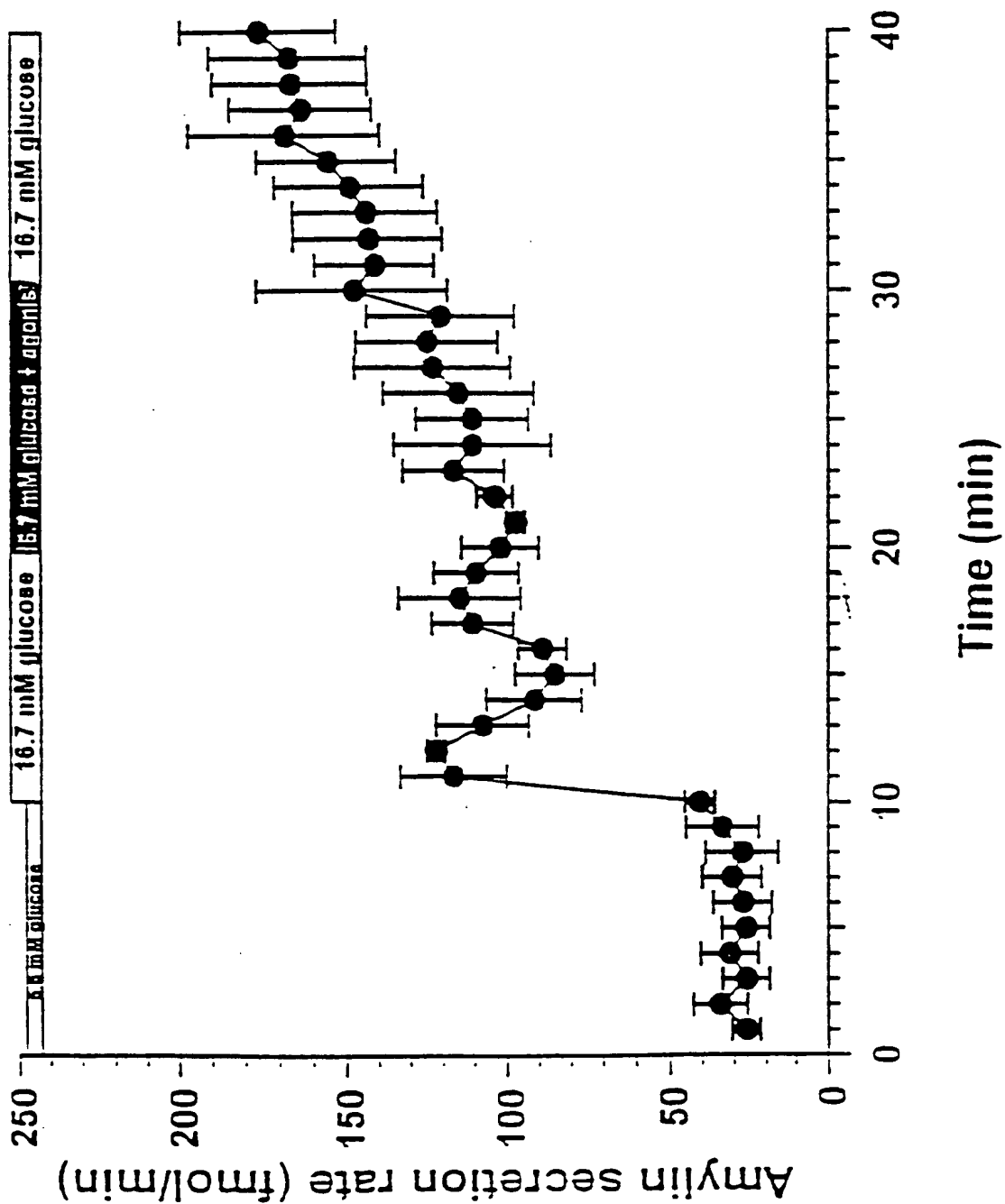


Fig. 2

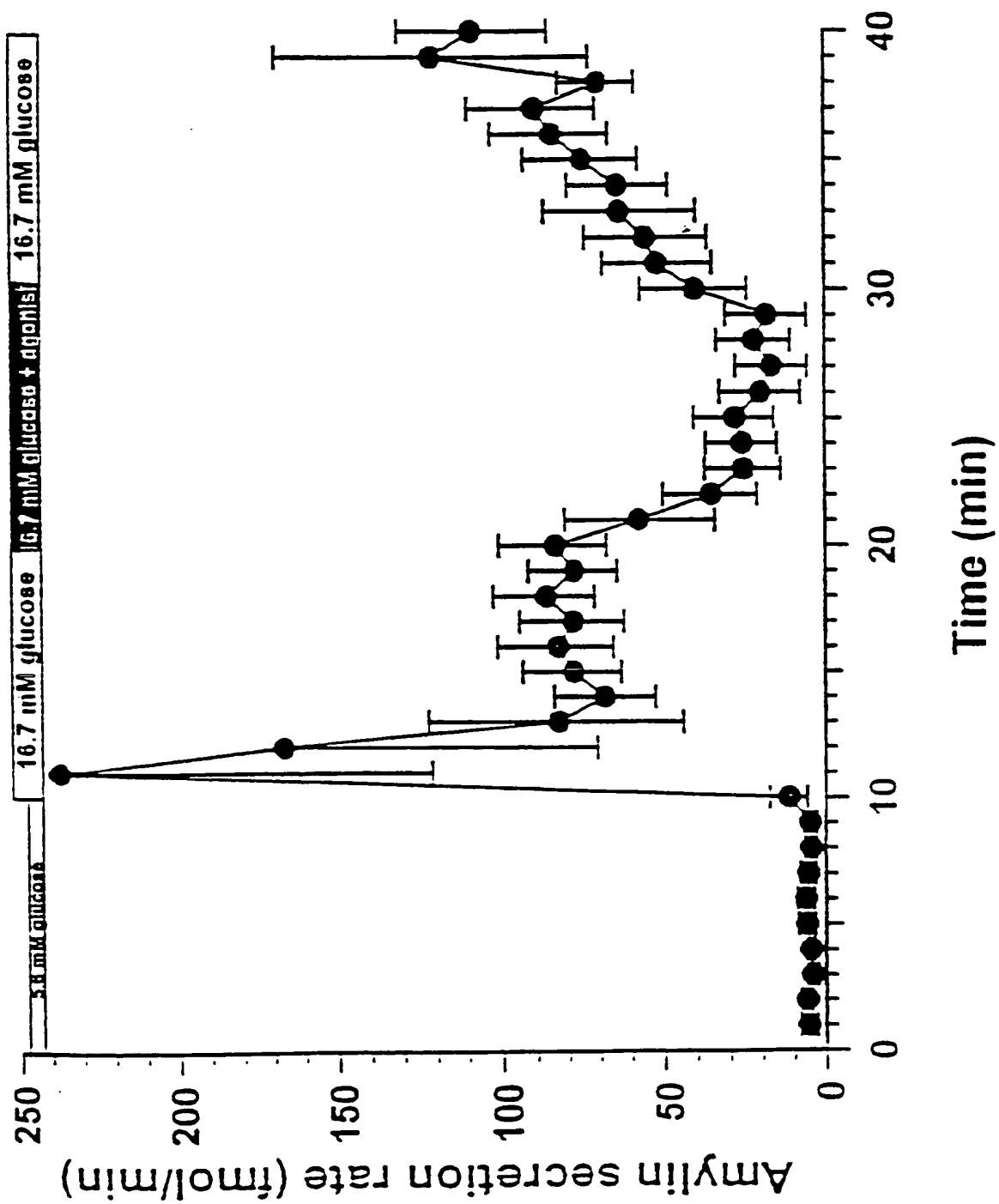


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02064

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 A61K38/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 13130 (ARCH DEV CORP) 8 July 1993 ---	
A	HORM .METAB. RES., vol. 24, no. 6, 1992, pages 251-253, XP000602469 K. INOUE ET AL.: "Effects of exogenous somatostatin and insulin on islet amyloid polypeptide (Amylin) release from perfused rat pancreas." ---	
P,A	JOURNAL OF SURGICAL RESEARCH, vol. 59, no. 1, July 1995, pages 85-90, XP000602464 S. MOLDOVAN ET AL.: "Somatostatin inhibits B-cell secretion via a subtype-2 somatostatin receptor in the isolated perfused human pancreas." --- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *A* document member of the same patent family

Date of the actual completion of the international search

20 September 1996

Date of mailing of the international search report

07-10-1996

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Cartagena y Abella,P

IN NATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02064

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>DIABETOLOGIA (SUPPL 1), vol. 38, September 1995, page A106 XP000602793 M. DAVENPORT ET AL.: "Somatostatin type 5 receptors mediate the inhibitory action of somatostatin on insulin secretion in zucker fatty rat perfused pancreas." -----</p>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 96/02064

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 13-15 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

IN NATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 96/02064

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9313130	08-07-93	US-A- 5436155	25-07-95
		AU-B- 661110	13-07-95
		AU-A- 3426993	28-07-93
		EP-A- 0621874	02-11-94
		JP-T- 7503611	20-04-95
